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ORIGINAL PAPER

Thomas M. Hohn · Anne E. Desjardins
Susan P. McCormick

The *Tri4* gene of *Fusarium sporotrichioides* encodes a cytochrome P450 monooxygenase involved in trichothecene biosynthesis

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Abstract The *Tri4* gene of *Fusarium sporotrichioides* was isolated from a cloned DNA fragment carrying the *Tri5* gene by complementation of a *Tri4*$^-$ mutant. The nucleotide sequence of *Tri4* was determined and the locations of three introns were identified. Analysis of *Tri4* mRNA levels revealed that transcription reached maximum levels coincidently with the onset of trichothecene biosynthesis, and then declined 20-fold over the next 8 h. Disruption of *Tri4* resulted in the loss of production of both trichothecenes and apotrichodiol and the accumulation of the unoxygenated pathway intermediate trichodiene. Transformants lacking a functional *Tri4* gene were able to convert isotrichotriol, an early pathway intermediate, to T-2 toxin suggesting that most pathway enzymes are present in *Tri4*$^-$ mutants. These data suggest that the enzyme encoded by *Tri4* catalyzes the first oxygenation step in the trichothecene pathway and participates in apotrichodiol biosynthesis. *Tri4* encodes a protein of 520 residues (M_r = 59 056) that shows significant homology with members of the superfamily of cytochromes P450. It appears most similar to the CYP3A subfamily (24.6% amino acid identity). Because it contains less than 40% positional identity with other cytochromes P450, the *Tri4* gene has been placed in a new cytochrome P450 gene family designated *CYP58*.

Key words *Fusarium sporotrichioides* · Trichothecene biosynthesis · Cytochrome P450 Apotrichothecene · Trichodiene

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T. M. Hohn (✉) · A. E. Desjardins · S. P. McCormick
Mycotoxin Research Unit, USDA/ARS, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, IL, 61604 USA

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Introduction

Trichothecenes constitute a large family of sesquiterpenoids that are produced by members of several genera of filamentous fungi including *Fusarium* and *Myrothecium* (Desjardins et al. 1993), and have been shown to accumulate in at least two species of the plant genus *Baccharis* (Jarvis 1992). Some trichothecenes are potent inhibitors of protein synthesis (McLaughlin et al. 1977) and are highly cytotoxic. The production of trichothecenes by some plant pathogens appears to enhance the virulence of these fungi on specific host plants (Desjardins et al. 1992, 1989).

The biosynthesis of most sesquiterpenoids begins with the cyclization of farnesyl pyrophosphate (FPP) to yield hundreds of different cyclic products. These cyclization reactions are catalyzed by members of the sesquiterpene synthase group of enzymes (Cane 1990). Most sesquiterpene synthase products are then modified by various oxygenation reactions. The parent compound of trichothecenes, trichodiene (Fig. 1), may undergo as many as seven different oxygenations during T-2 toxin (Fig. 1) biosynthesis (Desjardins et al. 1993). Trichodiene also undergoes multiple oxygenations in the biosynthesis of apparent shunt metabolites such as apotrichothecenes and sambucinol (Greenhalgh et al. 1989). In trichothecenes, specific oxygens such as the 12,13-epoxide have been shown to be required for toxicity (Colvin and Cameron 1986).

Cytochrome P450 monooxygenases have been characterized from a large number of organisms and are widely distributed in nature (Gonzalez and Nebert 1990; Nelson and Nebert 1993). However, information on cytochromes P450 from filamentous fungi is still limited (Attar et al. 1989; Kizawa et al. 1991; Maloney and VanEtten 1994; Van Gorcom et al. 1990). The involvement of cytochromes P450 in the biosynthesis of several plant cyclic terpenoids has been reported (Funk and Croteau 1994; Karp et al. 1987) but little is known concerning terpenoid oxygenation reactions in

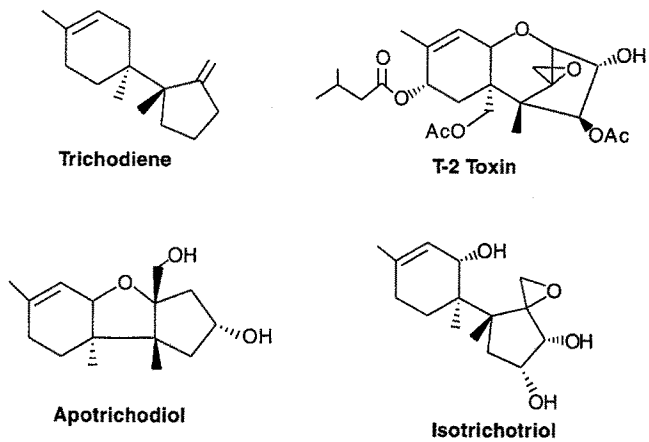

Fig. 1 Structures of T-2 toxin, apotrichodiol, isotrichotriol, and trichodiene

fungi. Recently, crude extracts from *Fusarium culmorum* were shown to oxygenate a trichothecene analog (Gledhill et al. 1991). Oxygenation of the trichothecene analog was found to be NADPH dependent, and inhibited by carbon monoxide, suggesting the participation of a cytochrome P450. Additional indirect evidence for the participation of cytochrome P450s in trichothecene biosynthesis comes from the observation that the oxygens in T-2 toxin other than those contributed by acetyl and isovaleryl esters appear to be derived from molecular oxygen (Desjardins et al. 1986).

Recently we reported that at least three genes involved in trichothecene biosynthesis are closely linked in *F. sporotrichioides* and that a DNA fragment carrying the *Tri5* gene complements a *Tri4*$^-$ mutant (Hohn et al. 1993). In this paper we report the characterization of the *Tri4* gene. Analysis of the deduced amino acid sequence of *Tri4* indicates that it is a cytochrome P450 monooxygenase belonging to a new P450 gene family.

Materials and methods

Materials

Complete sequencing of both DNA strands was performed using the Taq DyeDeoxy Terminator Sequencing kit (Applied Biosystems, Foster City, Calif.) with PCR product templates. Sequencing reactions were analyzed on the Applied Biosystems Model 373 automated DNA sequencer. All DNA probes were labeled with α-[32P]dCTP obtained from Dupont NEN Research Products, Boston, Mass, using the Prime-a-Gene System kit (Promega Madison, Wis.). The plasmid pGEM7Zf + was purchased from Promega.

Strains, media, and culture conditions

F. sporotrichioides NRRL 3299 was obtained from the USDA/ARS Culture Collection at the NCAUR, Peoria, Ill. Strain MB5493 (*Tri4-1*$^-$) was generated by UV mutagenesis (Beremand 1987) and is available from the Fusarium Research Center, Pennsylvania State University as T-915. Cultures were grown in YPG medium (0.3% yeast extract, 1% peptone, 2% glucose) for DNA isolation and in GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone) for trichothecene and RNA analyses (Ueno et al. 1975). All cultures were inoculated to a final concentration of 10^6 conidia/ml and incubated at 28° C on a gyratory shaker (200 rpm). Dry weight analysis was performed as described by Hohn and Beremand (1989). Competent cells of *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, Calif.) were used for all cloning procedures.

Polymerase chain reaction

PCR conditions were as described by Proctor and Hohn (1993) but with some modifications. Briefly, amplification reactions contained 50 mM KCl, 10 mM TRIS-Cl (pH 8.4), 2.5 mM $MgCl_2$, 100 μg/ml gelatin, 225 μM of each deoxyribonucleotide triphosphate, 0.5 pmol/ml of each primer (Table 1) and 0.025 U/μl AmpliTaq (Perkin-Elmer Norwalk, Conn.). Unless noted otherwise, each amplification cycle employed the following conditions: template denaturation for 1 min at 94° C; primer annealing for 1.5 min at 45–50° C; and primer extension for 2 min at 72° C (5 min last cycle). Most amplifications consisted of 25 cycles. PCR products were cloned directly into the commercially prepared cloning vector pCR2000 (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions.

Plasmid constructions and fungal transformation

For complementation studies the *Tri4* gene was amplified by PCR using primers 272 (5′-CGCGAGCTCGGTTCACATCTTCAACTG-3′) and 273 (5′-CGCGAGCTCTAGGAGACAGTATTGACG-3′), both of which contain a *Sac*I site. The resulting 2654 bp fragment was cloned into the *Sac*I site of the fungal transformation vector pUCH1 (Turgeon et al. 1987) to yield plasmid pTri4-2. For gene disruption the *Sma*I-*Xba*I fragment of pTri4-2, consisting of a doubly truncated portion of the *Tri4* gene, was cloned into pUCH1 to yield plasmid pTri4-1. Transformation of *F. sporotrichioides* and the subsequent selection and isolation of transformants were performed according to the procedure described in (Hohn et al. 1993).

Nucleic acid hybridizations

DNA isolation and Southern blotting were performed according to the protocol of Hohn and Desjardins (1992). To isolate RNA, cultures were grown in GYEP medium and harvested by filtration. The mycelial mats (approx. 0.5–1.0 g) were immediately ground in liquid N_2 and RNA was isolated with the RNaid kit (Bio 101, La Jolla, Calif.) and the acid phenol procedure described in the manufacturer's product literature. Northern blotting was carried out as described by Proctor and Hohn (1993) using a [^{32}P]-labeled DNA probe. Blots were analyzed using the AMBIS radioanalytical system (AMBIS, San Diego Calif.).

cDNA isolation

Synthesis of the *Tri4* cDNA coding sequence was performed with the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, Conn.) using random hexamers as primers for reverse transcription. The RNA template was prepared as described above and other reaction components and conditions were as specified by the manufacturer with the exceptions noted below. The primers for PCR were 275 (5′-GCGCCATGGTTGATCAAGACTGG-3′) and 274 (5′-GCGGAATTCACGGGTTGATGTAGTAGG-3′). The conditions

Table 1 Oligonucleotides used as primers for polymerase chain reactions

Primer number	Sequence[a,c]	*Tri4* location[b]	Target sequence
272	CFCGAGCTCGGTTCATCTTCAACTG	Not shown[d]	*Tri4* gene (5′ end)
273	CGCGAGCTCTAGGAGACAGTATTGACG	2114–2097	*Tri4* gene (3′ end)
275	GCGCCATGGTTGATCAAGACTGG	1–18	*Tri4* cDNA (5′ CDS[e])
274	GCGGAATTCACGGGTTGATGTAGTAGG	1799–1781	*Tri4* cDNA (3′ CDS)
397	TTCCGAGCACCAGCAGCAGTATAGATG	387–364	*Tri4* cDNA (5′ end)
396	CGCCTTAAGATAGGATGGATCCCTGATGTGAAC	355–332	*Tri4* cDNA (5′ end)
302	AATGTACCGCGAGACC	None	β-Lactanase gene
303	CAACAGCGGTAAGATCC	None	β-Lactamase gene
247	GGTCAACATGATGTCAGG	None	Promoter1 (5′ end)

[a] Restriction sites used for cloning are underlined
[b] Location within the sequence shown in Fig. 4
[c] Sequences are shown in the 5′ to 3′ orientation
[d] Located 161 bp upstream from nucleotide − 382 in Fig. 4
[e] Coding sequence of *Tri4*

for PCR were as described above. The resulting PCR product was extracted from an agarose gel band with Gene Clean (BIO 101 Inc., La Jolla, Calif.) and used as a template for a second PCR step using the two primers and conditions described above. The second PCR product was extracted as above and cloned into a plasmid derived from pGEM7zf + (pTA3) which had been modified to permit the generation of 3′ T overhangs following digestion with *Xcm*I (T. M. Hohn, unpublished). The 5′ end of the *Tri4* cDNA was obtained using the 5′ AmpliFINDER RACE kit (Clontech Laboratories, Palo Alto, Calif.) which employs a modification of the RACE protocol (Dumas et al. 1991). The *Tri4* directed primer for the initial amplification was 397 (5′-TTCCGAGCACCAGCAGTATAGATG-3′) while the nested primer for the second amplification was 396 (5′-CGCCTTAAGATAGGATGGATCCCTGATGTGAAC-3′).

Analysis of fungal transformants

All transformants were analyzed by PCR using primers 302 (5′-AATGATACCGCGAGACC-3′) and 303 (5′-CAACAGCGG-TAAGATCC-3′) to confirm the presence of the transformation vector. These primers are specific for the β-lactamase coding sequence present in pTri4-1 and pTri4-2. To confirm the disruption of the *Tri4* gene by pTri4-1 transformant DNAs were analyzed using primers 247 (5′-GGTCAACATGATGTCAGG-3′), and 275. Primer 247 corresponds to sequence present at the 5′ end of the Promoter1 sequence in pUCH1 which is adjacent to the insertion site for the disruption fragment in pTri4-1 while primer 275 corresponds to *Tri4* sequence not present in pTri4-1. Integration of pTri4-1 at the *Tri4* locus should result in the juxtaposition of these two primer sequences such that a 1553 bp fragment would be amplified.

Trichothecene analysis

Liquid cultures were analyzed for trichothecene toxins by gas-liquid chromatography (GLC). Samples (5.0 ml) from 25 ml YEPD cultures grown for 7 days were transferred to a test tube and mixed with 2.0 ml of ethyl acetate by vortexing (60 s). Following centrifugation, the organic layer was removed and 2 µl analyzed by GLC. Concentrations of trichothecenes were determined with the appropriate standard curves. Compound identifications were confirmed with gas chromatgraphy/mass spectrometry (GC/MS). Low-resolution mass spectra were obtained by GC/MS on a Hewlett Packard 5891 mass selective detector fitted with a DB-5-MS column (15 m × 0.25 mm film thickness).

Whole cell feeding experiments

Conidia were prepared by washing V-8 plates and then used to inoculate flasks (50 ml) containing 10 ml YEPD medium at a density of 5×10^4 conidia/ml. Cultures were incubated on a gyratory shaker (200 rpm) at 28° C. After 24 h, a 25 mM stock solution of the trichothecene in dimethylsulfoxide (DMSO) was added to the culture to a final concentration of 250 µM (1% DMSO). Control cultures had 1% DMSO added. Cultures were incubated on the rotary shaker at 28° C for an additional 5 days and were then extracted with ethyl acetate and analyzed by GLC.

Computer analyses

Comparative analyses involving protein data bases were performed with the FASTA (Pearson and Lipman 1988) and BLAST (Altschul et al. 1990) programs for the PIR 41.0 and Swiss-Prot 29.0 data bases. Alignments between individual sequences employed the program CLUSTAL within PCGENE (Intelligenetics, Moutain View, Calif.).

Results

Isolation and characterization of Tri4

Previously we found that a 7.1 kb genomic DNA fragment designated FSC13-9 (Fig. 2), carrying the *Tri5* gene was capable of complementing a UV-induced *Tri4*$^-$ mutant of *F. sporotrichioides* (MB5493) (Hohn et al. 1993). *Tri4*$^-$ mutants accumulate the

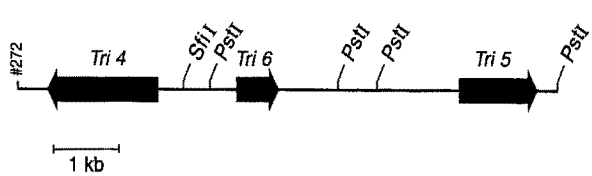

Fig. 2 Physical map of FSC13-9. The location of the *Tri4*, *Tri6*, and *Tri5* genes is shown. The direction of transcription is indicated by the *arrows*. The location of primer 272 is also shown

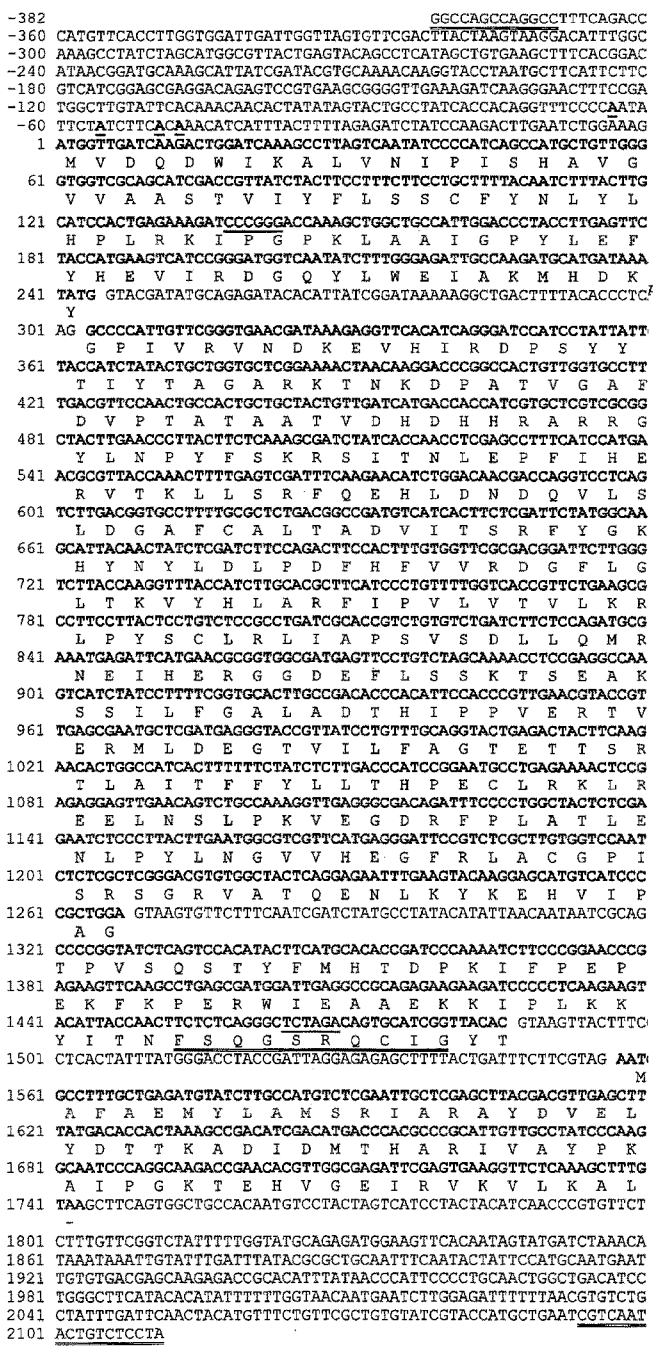

Fig. 3 Nucleotide sequence of the *Tri4* gene. Sequence features: (i) 5′ flanking sequence: *Sfi*I site at the 5′ end of sequence is *doubly underlined*, 5′ ends of four RACE products are indicated in *bold with underlining*; (ii) coding region: nucleotide coding sequence indicated in *bold* with sequence translation underneath, the amino acid code letter occurs under the middle nucleotide of the each codon, intron sequences are in *regular type* and separated from the coding sequence by a *space*, the *Sma*I and *Xba*I sites used for constructing the disrupter plasmid are *underlined*, the amino acid sequence corresponding to the conserved cysteinyl peptide involved in heme binding is indicated in *bold with underlining*; and (iii) 3′ flanking sequence: location of primer 273 is shown at 3′ end of sequence by *doubly underlining*. The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL data bank with accession number 422462

unoxygenated intermediate trichodiene but do not accumulate trichothecenes or apotrichodiol (3α, 13-dihydroxy-epiapotrichothec-9-ene). Sequence analysis of this fragment revealed a 1106 nucleotide open reading frame transcribed in an orientation opposite to that of *Tri5*. Further analysis indicated the presence of several introns based on the established intron consensus sequences for filamentous fungi (Rambosek and Leach 1987). Using primers 272 and 273 a 2654 bp fragment was amplified and cloned into the fungal transformation vector pUCH1 (Turgeon et al. 1987). The resulting plasmid, designated pTri4-2, was used to transform the *Tri4*⁻ mutant. Of the seven transformants analyzed six were restored to trichothecene production. The trichothecene profiles of these cultures were similar to those of *F. sporotrichioides* NRRL 3299 indicating that pTri4-2 contains at least a portion of the *Tri4* gene. The nucleotide sequence of the DNA fragment inserted into pTri4-2 is shown in Fig. 3, with the exception of 161 nucleotides upstream from the *Sfi*I site. This upstream sequence has recently been determined as part of the 5′ flanking region for the *Tri6* gene (Proctor et al. 1995).

Using primers 275 and 274, a cDNA representing the *Tri4* coding region was amplified and sequenced. It contained an open reading frame of 1560 bp encoding a protein sequence of 520 amino acids with a predicted molecular weight of 59 056 Da. Comparison of the cDNA and genomic sequences confirmed the existence of three introns (Fig. 3). The 5′ cDNA sequence was amplified with primers 396 and 397 using a modification of the RACE method (Dumas et al. 1991). Following PCR a single band was observed, however, sequence analysis of four cloned PCR products revealed that they all started at different nucleotide positions between −48 and −64 bp.

To characterize further the *Tri4* gene, mutants were made by molecular disruption with a plasmid (pTri4-1) containing a doubly truncated fragment of the predicted *Tri4* coding region. The disrupter plasmid was constructed by cloning the 1327 bp *Sma*I- *Xba*I fragment internal to the coding region of *Tri4*, into pUCH1. Following transformation, 27 hygromycin B-resistant colonies were isolated and shown by PCR to carry at least a portion of pTri4-1. To determine if integration of pTri4-1 had occurred at the *Tri4* locus, transformants were analyzed by PCR with primers specific for the disruption vector (247) and a region of the *Tri4* coding sequence not present in pTri4-1 (275). Three transformants (E9, E11, and F15) were found to produce a fragment of the same size (1553 bp) as that predicted for the integration of pTri4-1 at the *Tri4* locus. These same three transformants also had unique trichothecene profiles as determined by GC analysis of culture extracts. The trichothecene production phenotypes for E9, E11, and F15 were identical to that of the UV induced *Tri4*⁻ mutant MB5493 while the other

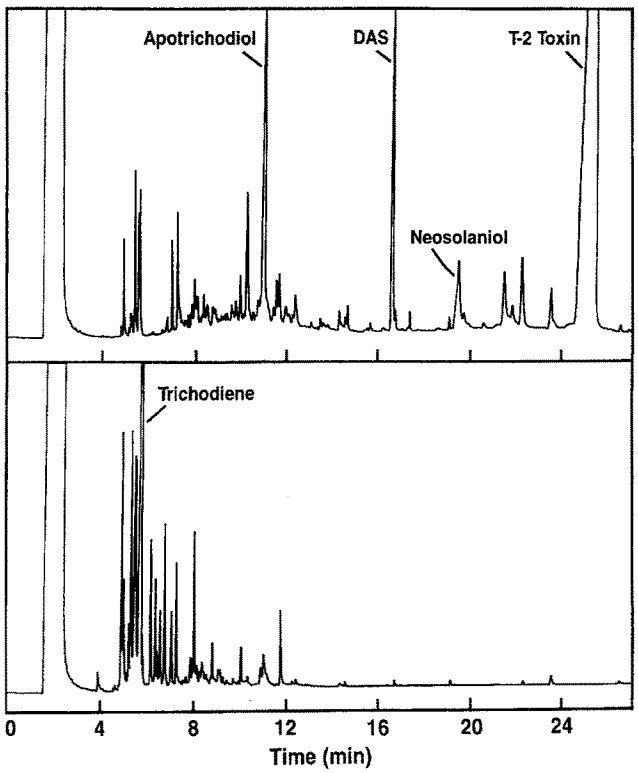

Fig. 4 Chromatogram of *Fusarium sporotrichioides* NRRL 3299 (*Tri4*$^+$) and F-14 (*Tri4*$^-$) culture extracts analyzed by gas-liquid chromatography. Cultures were grown on GYEP medium for 7 days. Top panel; *F. sporotrichioides* NRRL 3299 chromatogram; the locations of the T-2 toxin, diacetoxyscirpenol, neosolaniol, and apotrichodiol peaks are indicated. Bottom panel; F-14 chromatogram; the location of the trichodiene peak is indicated.

transformants were indistinguishable from *F. sporotrichioides* NRRL 3299. Extracts from all three *Tri4*$^-$ transformants contained elevated levels of trichodiene but lacked trichothecenes and apotrichodiol.

Conversion of pathway intermediates to T-2 toxin in the *Tri4*$^-$ mutant

The primary end product of the trichothecene pathway in *F. sporotrichioides* NRRL 3299 is T-2 toxin which constitutes 60–80% of the total trichothecenes produced in GYEP-grown cultures. Two less oxygenated trichothecenes, 4,15-diacetoxyscirpenol and neosolaniol, are also produced in small amounts. All three of these trichothecenes are absent from *Tri4*$^-$ cultures grown in GYEP medium (Fig. 4), as determined by GC analysis of culture filtrates. Because the *Tri4* gene product catalyzes only the second committed step in the trichothecene pathway, efforts were made to determine if later pathway enzymes were present in a *Tri4*$^-$ transformant. The presence of later pathway enzymes in transformant F-15 was determined by feeding an early pathway intermediate, isotrichotriol (Fig. 1), and then analyzing culture extracts for T-2 toxin production. Conversion of isotrichotriol to T-2 toxin by cultures of F15 was approximately 30%. In addition, F15 cultures were also able to efficiently convert nine other pathway intermediates to T-2 toxin.

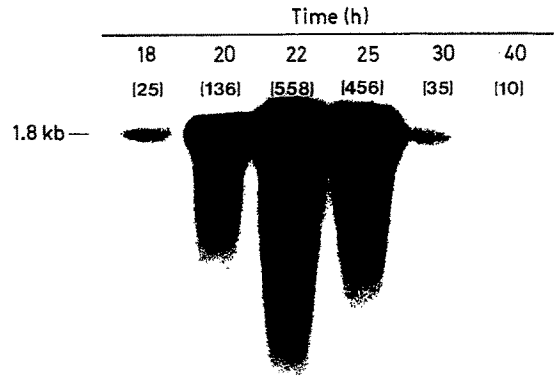

Fig. 5 Northern blot probed with the *Tri4* coding region. The numbers above the bands represent cpm, determined by the AMBIS radioanalytical system

Transcription of the *Tri4* gene

Cultures of *F. sporotrichioides* NRRL 3299 grown in GYEP medium begin to produce trichothecenes at 22–25 h post-inoculation (Hohn and Beremand 1989). The transcription of *Tri4* was investigated in GYEP cultures incubated for 18–30 h. Northern blot analysis of total RNA (Fig. 5) revealed a single band of approximately 1.8 kb in cultures grown for 18, 20, 22, 25, and 30 h. The *Tri4* transcript was barely detectable at 18 h, increased to maximum levels at 22 h, and then decreased 20-fold over the next 8 h.

Comparisons between *Tri4* and cytochrome P450 sequences

In an effort to determine the function of the *Tri4* gene product (TRI4) comparisons were performed between TRI4 and sequences present in two protein data bases (PIR and Swiss-Prot). Sequence comparisons employed algorithms for both local (BLAST) and global (FASTA) alignments (Altschul et al. 1990; Pearson and Lipman 1988). The results from both types of alignments indicated that TRI4 was most closely related to cytochrome P450-type enzymes. Global alignments with individual P450 sequences revealed that TRI4 was most similar to the CYP3A subfamily with 24.6% identity to rabbit CYP3A6. A somewhat lower degree of similarity (22.6% identity) was also observed with another filamentous fungal enzyme, pisitin demethylase (CYP57) from *Nectria haematococca*. Sequence comparisons between CYP3A6, CYP57, and CYP53,

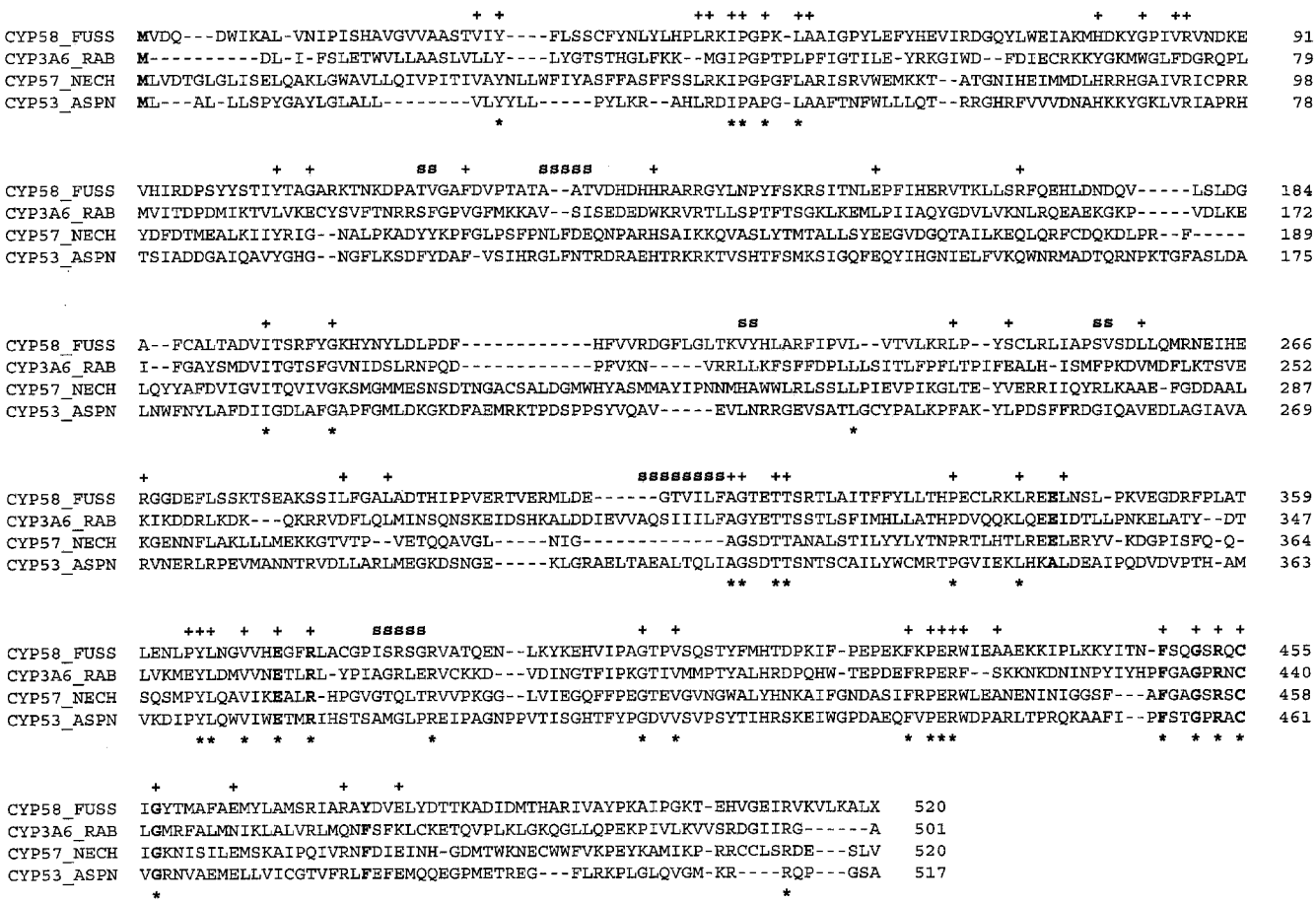

Fig. 6 Comparisons between *F. sporotrichioides* CYP58 and selected P450 sequences: CYP3A6 from rabbit (PIR A34236), CYP57 from *Nectria haematococca* (Swiss-Prot P38364), and CYP53 from *Aspergillus niger* (Swiss-Prot P17549). *Asterisks* indicate residues conserved in all sequences; +, for residues conserved in alignments between the filamentous fungal enzymes; *ss* (*sss*, etc.) for substrate recognition regions according to Gotoh (1992)

a cytochrome P450 from *Aspergillus niger*, are shown in Fig. 6.

Although the global sequence identity of TRI4 was less than 25% in comparison to cytochrome P450 sequences, much higher identity was observed using BLAST with some P450s over shorter sequences. For example, there are three regions of TRI4 that were found to have greater than 40% identity with CYP57 from *N. haematococca*. The 42 amino acid sequence starting at Ala^{314} and the 33 amino acid sequence starting at Phe^{448} both have 42% identity, while the 34 amino acid sequence starting at Val^{23} has 44% identity with the corresponding sequences in CYP57. The second region includes the heme binding domain and the third region overlaps the N-terminal region corresponding to the probable membrane anchor. Within the third region there is a remarkable 12 amino acid sequence with 83% identity starting at Leu^{43}. Low levels of sequence identity were observed for those sequences corresponding to the substrate recognition regions that have been proposed for the CYP2 family (Gotoh 1992).

In addition to containing regions with significant homology to individual cytochrome P450s, TRI4 also contains a number of residues that appear to be conserved in all P450s. The sequence motif that constitutes the heme binding domain of cytochrome P450s contains several residues that are highly conserved. One of these residues is a cysteine that serves as the fifth ligand for the coordination of heme iron at the monooxygenase active site. TRI4 contains a 10 amino acid sequence starting at Phe^{449} in perfect agreement with the heme binding domain consensus sequence. Further, this sequence is aligned with the corresponding sequence in alignments with other cytochromes P450. Based on these sequence alignments it is likely that Cys^{465} functions as the fifth ligand in the heme binding domain of TRI4. Several other residues are also found to be highly conserved in P450s although their functions are unknown (Nelson and Strobel 1988, 1989), the corresponding residues in TRI4 are Glu^{343}, Glu^{372}, and Arg^{375}.

Discussion

Several genes involved in trichothecene biosynthesis are clustered in *F. sporotrichioides* (Hohn et al. 1993).

We have identified the *Tri4* gene of *F. sporotrichioides* on a cloned DNA fragment and shown that it is located immediately upstream from two previously characterized pathway genes, *Tri5* and *Tri6*. Localization of *Tri4* to a 2654 bp fragment was demonstrated by complementation of a *Tri4*$^-$ mutant and then confirmed by gene disruption. *Tri4* transcription was found to occur in a direction opposite to both *Tri5* and *Tri6* transcription.

Based on nucleotide sequence analysis of the *Tri4* gene and *Tri4* cDNA, three introns were identified within the 1743 bp coding region. The 5′ ends of four cloned cDNAs amplified by the RACE method were sequenced and found to start at different nucleotides within a 20 bp segment of the leader sequence of the *Tri4* transcript. This result may reflect the existence of multiple transcriptional start sites or could be due to heterogeneity in the mRNA template resulting from RNA degradation. In either case, it shows that transcription can be initiated at least 63 bp upstream from the ATG.

Several lines of evidence indicate that *Tri4* encodes a cytochrome P450 monoxygenase. Firstly, both global and local sequence alignments with proteins in the PIR and Swiss-Prot data bases indicate that TRI4 is closely related to cytochrome P450s. Secondly, in global alignments a number of highly conserved residues in P450s are aligned with the corresponding residue in TRI4. These include the conserved residues that are part of the cysteinyl peptide involved in heme binding. Thirdly, disruption of *Tri4* results in an altered trichothecene production phenotype characterized by the accumulation of trichodiene, the only known non-oxygenated pathway intermediate. Additional evidence for the participation of cytochromes P450 in trichothecene biosynthesis comes from studies showing that in *F. sporotrichioides* all oxygenation reactions involving carbon atoms derived from trichodiene employ molecular oxygen (Desjardins et al. 1986), and that cultures of *F. sporotrichioides* treated with ancymidol, a potent inhibitor of many cytochromes P450, accumulate trichodiene but no trichodiene metabolites (Desjardins et al. 1987). Together, these data strongly suggest that *Tri4* encodes a cytochrome P450 monooxygenase that catalyzes the initial oxygenation reaction with trichodiene in trichothecene biosynthesis and participates in apotrichothecene biosynthesis. Because TRI4 shares $< 40\%$ positional identity with any characterized member of the cytochrome P450 superfamily it therefore represents the first member of a new family. TRI4 has been given the name CYP58 by the cytochrome P450 Nomenclature Committee (D.W. Nebert, personal communication).

The ability of *Tri4*$^-$ mutants to convert a variety of pathway intermediates to T-2 toxin indicates that most pathway enzymes are present. This result is consistent with *Tri4* encoding a pathway enzyme that is not required for the activity of later pathway enzymes. The accumulation of trichodiene in *Tri4*$^-$ mutants demonstrates that trichodiene synthase activity is also independent of a functional *Tri4* gene product.

Northern blot analysis indicated that the levels of *Tri4* transcript in GYEP cultures increased to maximum levels at 22 h and then decreased 20-fold by 30 h. This result is consistent with a recent report that the expression of *Tri4* is positively regulated by the *Tri6* gene product and that *Tri6* expression decreases to low levels between 25 and 30 h (Proctor et al. 1995). Because trichothecene accumulation in GYEP medium is known to be linear with respect to time between 25 and 120 h (Hohn and Beremand 1989), these data suggest that either the *Tri4* transcript levels increase at some time after 30 h or that the product of this gene is stable for extended periods of time in stationary phase cultures. Expression of *Tri5* has also been shown to decrease approximately 20-fold between 22 and 30 h (Proctor et al. 1995). This change in *Tri5* message levels is accompanied by a much smaller decrease in enzyme activity of 25% (Hohn and Beremand 1989), suggesting that enzyme stability plays an important role in regulating TRI5 activity.

Identifying the earliest intermediates in the trichothecene pathway has proven to be difficult. Early intermediates do not appear to accumulate using either solid or liquid culture methods and have not been detected using various labeled precursor feeding techniques. In addition, no mutants have been found for genes representing the steps between trichodiene and decalonectrin. Isolation of the *Tri4* gene provides an opportunity to characterize the first trichodiene metabolite in the trichothecene pathway. Heterologous expression of *Tri4* in fungal hosts such as yeast or *Aspergillus nidulans* may permit the identification of the TRI4 product.

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